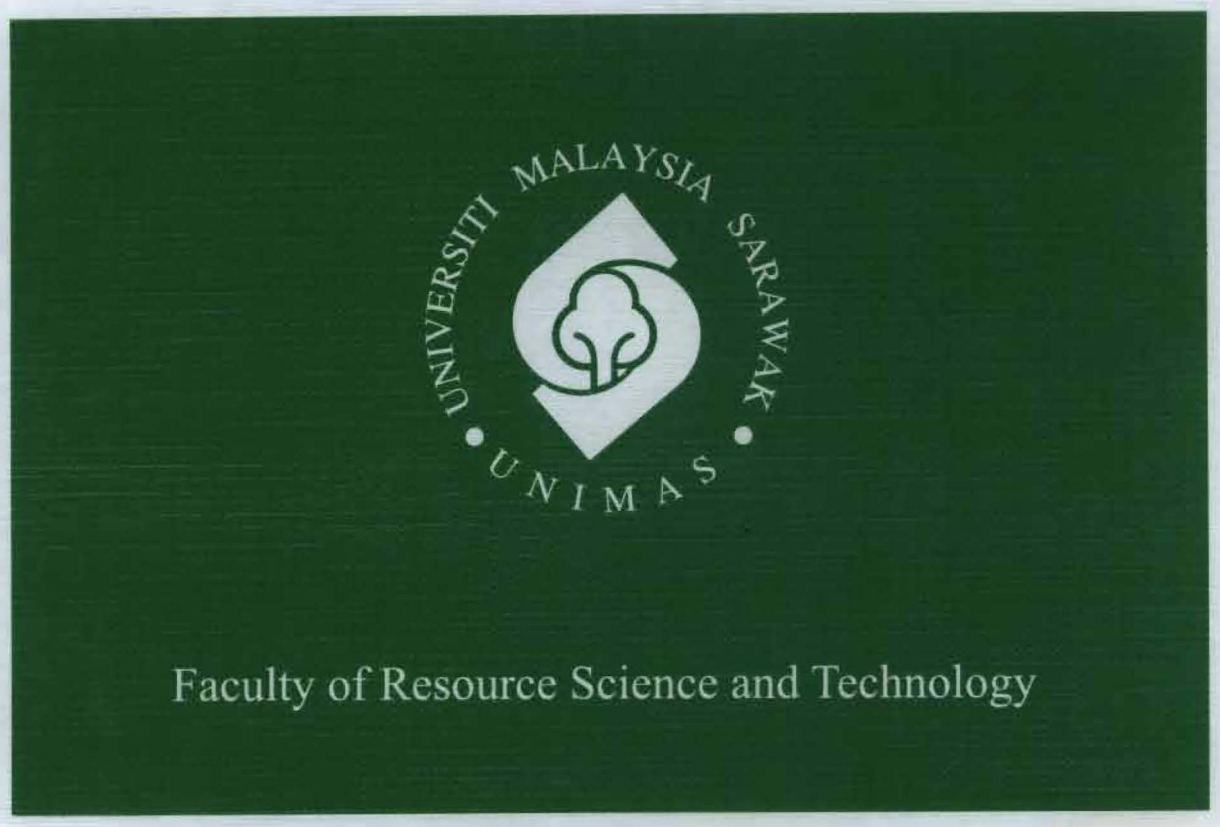


# Ethanol fermentation using isolated *Candida tropicalis* ATTCCa under non-conventional temperatures

**Lim Ming Gim**
**23867**



**Bachelor of Science with Honours**
**(Biotechnology Resource)**
**2012**

# Acknowledgement

First of all, and most importantly, I would like to glory God for His blessings and guidance throughout the projects. I also would like to express my thankfulness to my family especially my mother for her support and advice during undertaking this project.

I also would like to express my gratefulness and sincere thanks to my supervisor, Associate Professor Dr. Cirilo Nolasco Hipolito for spending much of the time for guidance, supervision and patience during the way to complete this project. Besides that, I would also like to express my appreciation to my Co-Supervisor Professor Dr. Kopli bin Bujang for his assistance in any aspect of the project.

I also would like to express my thankfulness to Ms. Rubena Malfia Kalmaldin, Ms. Nurul Faseeha, Ms. Sarina, Ms. Nurul Jannah, and Ms. Kommathi for their generous help and valuable help and advice especially every time I encountered the problems in this project.

Last but not least, I also would like to thanks to my lab-mates and friends, Amirul, Afri, Naimmi, Surya, and Andy Lau for their support, caring and concern since the beginning of this project.

# Declaration

**UNIVERSITI MALAYSIA SAWARAK**

Grade: _____

**Please tick (√)**

Final Year Project Report ☑

Masters ☐

PhD ☐

## DECLARATION OF ORIGINAL WORK

This declaration is made on the **3rd day of July 2012.**

**Student's Declaration**:

I **Lim Ming Gim, 23867, Faculty of Resource Science and Technology** hereby declare that the work entitled, "**Ethanol fermentation using *Candida tropicalisis* ATTCCa under non-conventional temperatures"** as my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

**3rd July 2012**
Date submitted

**Lim Ming Gim (23867)**
Name of the student (Matric No.)

**Supervisor's Declaration:**

I **Cirilo Nolasco Hipolito** hereby certifies that the work entitled, "**Ethanol fermentation using *Candida tropicalisis* ATTCCa under non-conventional temperatures"** was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of Bachelor of Science with honours, and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: **Cirilo Nolasco Hipolito** (Name of the supervisor) Date: **3rd July 2012**

I declare this Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

**Validation of Project/Thesis**

I therefore duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitise the content to for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student itself neither third party on this Project/Thesis once it becomes sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student's signature _____

3/7/2012 (Date)

Cirilo Nolasco Hipolito
Associate Professor
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
Supervisor's signature: _____

3/07/2012 (Date)

Current Address:

_____

_____

_____

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument was duly prepared by The Centre for Academic Information Services]

# Table of Contents

# List of Abbreviations

| | |
|---|---|
| CFU | Colonies Forming Units. |
| DCW | Dry Cell Weight |
| DNS | 3, 5-Dinitrosalicylic acid |
| g | Gram |
| g/L | Gram per Litre |
| L | Litre |
| h | Hours |
| HPLC | High-Performance Liquid Chromatography |
| HSS | Hydrolyzed Sago Starch |
| min | Minutes |
| OD | Optical Density |
| rpm | Revolution per Min |
| $^{o}C$ | Degree Celsius |
| RBF | Repeat Batch Fermentation |
| µl | Micro-litre |
| µ | Specific Growth Rate |

# List of Figures and Tables

# Ethanol fermentation using isolated *Candida tropicalis* at not conventional temperatures

**Lim Ming Gim**

Biotechnology Resource Programme, Department of Molecular Biology

Faculty of Resource Science and Technology

University Malaysia Sarawak

## ABSTRACT

*Candida tropicalis* isolated from rotten pineapple in Malaysia was used to perform ethanol fermentation in hydrolyzed sago starch. The effect of the temperature on the growth of the yeast was studied at 42 and 45°C using batch fermentation mode. From the study, this strain can considered as thermo tolerant yeast due to ability to growth well at 42°C and even to survive at 45°C growing at lower rate. Repeated batch fermentation RBF mode using hydrolyzed sago starch was performed to improve the productivity of ethanol at different concentration of 30g/L, 40g/L, 50 g/L, 60g/L, 90g/L and 100g/L for 10 fermentation cycles. In addition, the kinetics of ethanol production, glucose consumption, and by-products of the fermentation was analyzed during the RBF. The rate of glucose consumption of this strain during RBF at 42°C was low. This phenomenon was avoided by lowering the temperature from 42°C to 36°C and consequently high concentration of ethanol was achieved.

Key words: *Candida tropicalis*, ethanol, high temperature effects, repeat batch fermentation.

## ABSTRAK

*Candida tropicalis* yang diekstrak daripada nanas reput di Malaysia digunakan untuk menjalankan fermentasi dengan menggunakan substrat hidrolisis kanji sagu. Kesan suhu ke atas pertumbuhan yis yang telah dikaji adalah 42 dan 45 ° C dengan menggunakan fermentasi kelompok. Hasil kajian menunjukkan, strain ini boleh dianggap sebagai yis tahan haba kerana keupayaannya untuk hidup pada 42°C manakala pada 45°C didapati pertumbuhannya berkurang. Setelah mendapat suhu optimum di antatra 42°C dan 45°C, fermentasi 'repeated batch' (RBF) dijalankan bagi tujuan meningkatkan produktiviti etanol pada kepekatan hidrolisis kanji sagu yang berbeza iaitu 30g /L, 40g /L, 50g/L, 60g / L, 80g / L dan 100g /L untuk 10 kitaran. Di samping itu, kepekatan etanol, baki glukosa, dan produk sampingan juga dianalisis semasa RBF. Kadar penggunaan glukosa bagi strain ini semasa RBF pada 42°C didapati rendah. Fenomena ini boleh dielakkan dengan mengurangkan suhu fermentasi dari 42°C kepada 36°C. Dengan ini, etanol yang berkepekatan tinggi dapat dihasilkan.

Kata kunci: *Candida tropicalis*, etanol, kesan suhu tinggi, ulangi kelompok penapaian.

## 1.0 INTRODUCTION

Today's world is facing two critical problems: high fuel prices, and climatic change. Experts suggest that current fossil fuel would suffice to last only a few more decades (Demirbas, 2009). High usage of fossil fuel also causes its depletion in future (Demirbas, 2009). The combination of rising oil prices, the issue of security, and the climatic changes are propelling governments around the worlds to enact powerful incentives for the use of non-fossil fuel. Hence, there has been widespread recent interest in learning more about obtaining fuels from non-fossil sources (Demirbas, 2009). Due to it could be an environmental friendly bio-fuel, ethanol is gaining increased public and scientific attention. Bio-ethanol is an alcohol made by fermentation mostly from carbohydrate such as starch or sugars. To provide the numerous environmental and social benefits resulting from the replacement of petroleum-based automotive fuels, it will be necessary to produce bio-ethanol efficiently from starchy materials using microorganisms (Fukuda, *et al.*, 2009). A number of organisms including fungi, yeast and bacteria have been screened for ethanol fermentation. Extensive studies have been carried out on the fermentation process of ethanol by these organisms, especially through yeast cells (Bajaj *et al.*, 2001).

Fermentation is one of the oldest and remains most economical methods of producing products highly acceptable to human (Steinkraus, *et al.*, 1983). Fermentation at high temperature using thermo-tolerant yeast can increase the ethanol productivity (Fernandez, *et al.*, 2003). There are several potential benefits associated with fermentation at high temperature; one of the benefits is that it is more favourable from an economics point of view due to the higher productivity of fermentation under vacuum (Lee, *et al.*, 1993). Banat *et al.*, (1998) reported that ethanol fermentation at high temperature using thermo-tolerant yeast

have benefits such as energy savings through reduced both cooling costs and contamination. Ethanol fermentation at high temperature also made simultaneous saccharification and fermentation more efficiency (Nonklang et al., 2008). There are some reports in the literature showing that some species of yeast can survive and growth at temperature exceeding 40°C (Szczodrak & Targonski, 2004). However, only few reports appear to be claim high-productivity of ethanol production by such organisms at high temperatures. For instance, in some studies conducted on the subject, the researches achieved 90% of ethanol yields higher temperatures than 40°C using strains of *Saccharomyces uvarum*, *Saccharomyces cerevisiae* and *Candida tropicalis* (Laluce *et al.*, 1993). Another study also show that their yeast species produced high level of ethanol after 24 hours of fermentation and the yeast cell still maintain it viability at 80% by D'Amore *et al.*, (1988).

In this study, a strain of thermo tolerant yeast- *Candida tropicalis* isolated from rotten pineapple was used to test its ability to produce ethanol at high temperature. To improve the productivity, the repeated batch fermentation mode was applied to reuse the cells in subsequent fermentation cycles.

## 2.0 OBJECTIVES

General: To perform repeated batch fermentation for ethanol production at high temperature using *Candida tropicalis* to improve the process productivity.

1. To study the effect of the temperature on the growth of *Candida tropicalis*.
2. To determine the kinetics of the fermentation as glucose consumption and ethanol production of *Candida tropicalis* during repeated batch fermentation at high temperature.
3. To study the other by-products produced by *Candida tropicalis* during repeated batch fermentation at high temperature.

## 3.0 HYPOTHESIS

*Ho: Candida tropicalis* is not able to stand high temperature for growing and to perform fermentation to produce ethanol.

Ha: *Candida tropicalis* is able to stand high temperature for growing and to perform fermentation to produce ethanol.

# 4.0 LITERATURE REVIEW

## 4.1 Ethanol as bio-fuel

Ethanol, also known as ethyl alcohol with a molecular formula $C_2H_5OH$ is a clear, colourless liquid with characteristic and agreeable odour (Mariam *et al.*, 2009). In dilute aqueous solution, it has sweet flavours, but in more concentrated solutions it has a burning taste (Patil, 1991). The melting point and boiling point of ethanol are -114°C and 78.5°C respectively and has a density of 0.789 g/ml at 20°C (Kaur & Kocher, 2002).

Ethanol is an important industrial chemical with emerging potential as a bio-fuel to replace fossil fuel (Rakin *et al.*, 2009). Bio-fuel is a type of bio-energy fuel whose energy is derived from biological carbon fixation. They include fuels derived from biomass conversion, as well as solid biomass, liquid fuels and various biogases. Bio-ethanol provides an alternative to fossil fuel dependency and emits fewer pollutants (Carvalho *et al.*, 1993). There are several route which can be distinguished to produce bio-fuel: extraction of vegetable oils, fermentation of sugars to alcohol, gasification and chemical synthesis and direct liquefaction (Hamelinck & Faaij, 2005). In present, bio-ethanol fuel covers 9-14% of the global demand, most of which as traditional, low-tech and inefficient cooking and heating in developing countries (Hall *et al.*, 1993). Countries such as Brazil and United State produce bio-ethanol fuel energy act as fuel for heat, electricity and transportation (Turkenburg, 2000).

There are several reasons important of bio-fuel acts as substitute for the fossil fuel; one of the reasons can seem in transportation. Transportation represents about 27% of the world

secondary energy consumption and is almost exclusively fuelled by fossil fuel. The share may increase to 29%-32% in 2050 (Intergovernmental Panel on Climate Change, 2000). The rapidly increasing demand for transportation fuels is combined with rapidly decreasing mineral fossil oil reserves of non-OPEC states. This increases dependency on a limited number of oil-providing countries (Rogner, 2000). According to International Energy Agency, in present bio-fuel provided 2.7% of the world's transport fuels and it have potentials to meet more than a quarter of world demand for transportation fuels by 2050. Hence, it is important to produce large amount of bio-ethanol fuel to substitute fossil fuel to solve this kind of problems. Furthermore, by the year 2040, it is estimated approximately half of the global energy supply will come from bio-fuel, and the electricity generation using bio-fuel will be more than 80% of the total global electricity production (Demirbas, 2009).

### 4.2 Ethanol production by yeast

Archaeological evidence proved that yeast has been used in Egypt as early as 4000 B.C. act as brewing agent to produce ethanol by the sugars fermentation (Yim & Glover, 2003). In present, yeasts were used extensively in batch fermentation to convert the sugars to ethanol for the production of alcoholic beverages and bio-fuel (Dombek & Ingram, 1987). This is due to yeasts has an ability to increase their rate of glycolysis and ethanol production under optimal conditions by producing more than 50 mmol of ethanol per hour per g of cell protein (Dombek & Ingram, 1987). Nowadays, more than 90% of ethanol produced per year is made using yeast.

Yeast fermentation performs strictly under anaerobic conditions, glucose split into two molecules of 3 carbon sugars through the glycolysis. Next an enzyme from yeast, Zymase changes the simple sugars into ethanol and carbon dioxide. Theoretically, 1g of glucose can

produce 0.51 gram of ethanol and 0.49 gram of carbon dioxide. Previous studies by scientists shown that glucose from sugarcane, fruits, sweet potato and agricultural wastes are suitable for fermentation (Nigam *et al.*, 1998).

### 4.3 Classification and characteristic of yeast

Yeasts are classified as unicellular eukaryotic microorganisms under the kingdom of fungi. There are around 100 genera and 700 species of yeasts. (Maragatham & Panneerselvam, 2011). Classification of yeasts up to species level can be done based on their morphological and physiological/biochemical characteristics (Mushtaq *et al.*, 2006). Generally, yeasts can divide into two main classes of fungi which are ascomycetes and basidiomycetes (Kurtzman, 1990). Feldmann (2005) reported that cell size, colour and shape of yeast have display a great diversity of yeast. Due to alteration of physical and chemical environment, yeast strain from a single species can display different morphology and colour.

### 4.4 Thermo-tolerant yeast-*Candida tropicalis*

There are different definitions of thermo-tolerant yeast. According to Laluce, *et al.*,1993, thermo-tolerant yeast are those yeast can survives in the temperature higher than 37°C while Slapack *et al.* (1987) defined that yeast can be classified as thermo-tolerant if they can grow at 40°C. Generally, temperatures of 36°C or above are considered high temperature for most of the species of yeast including *Candida tropicalis* (Nolasco Hipolito C., 2012).

*Candida tropicalis* is diploid ascomycetes yeast under the Candida genus (Desnos-Ollivier *et al.*, 2008). As reported by Kregar-van Rij (1984), approximately 165 species including *Candida tropicalis* have been identifies under Candida genus. *Candida tropicalis* can growth in ranging 23-51°C and growth best at 33°C (Nolasco Hipolito C., 2011). These species of vegetative cells generally show multilateral budding either in form of pseudohyphae or true hyphae which can stimulate under the anaerobic conditions. Under the direct examination, their hyphae show distinct point of constriction which resembling the sausage links.

### 4.5 Carbon sources for fermentation

Generally, the main sources of sugar required to produce ethanol are derived from energy crops (Ibeto *et al.*, 2011). These crops include, corn, maize, wheat, waste straw, willow, poplar trees, sawdust, reed canary grass, cord grasses, *Jerusalem artichoke*, *miscanthus* and sorghum plants (Coppola *et al.*, 2009). Corn, sugarcane, sweet sorghum and sweet potatoes are common carbon sources used in ethanol production (Ibeto *et al.*, 2011).

Due to its greater weight production each year around the world, corn is suitable for cultivation as a carbon source. Most of the corn components contain cellulose, a rigid material digested only by fungi and certain species of bacteria (Ibeto *et al.*, 2011). Of the 692 million metric tons of corn produced annually around the world, 280 million metric tons are produced by United States for growing of short-rooted corn mainly for ethanol production (Grassi, 2001).

Bio-ethanol production using sugarcane was started in Brazil and United States in the early 1970's (Chatanta *et al.*, 2008). The bagasse is the name given to the biomass that remains from the sugar stalk after it has been crushed and the sugar and garapa (juices) have been extracted. Although bagasse is not yet commercially converted into ethanol, however research works are on-going to profitably convert the bagasse into ethanol. Many sugar mill have utilized the bagasse for co-generation both heat and electric energy production (Ibeto *et al.*, 2011). Often in surplus, sugarcane proved to be an ideal source of fuel once it became profitable to mass-produce the ethanol (Grassi, 2001).

Sweet sorghum has capacity to provide a very wide range of renewable energy products. Their sugars contents consist of sucrose, fructose and glucose. Most of these sugars distributed in the stalks and only 2% in the leaves and panicle (Grassi, 2001). The stalks of sweet sorghum harvested just before flowering contain as much sugar as sugarcane (16-23 % Brix) (Ibeto *et al.*, 2011). Sweet sorghum is an excellent source of biomass as it yields 30-35 tones of biomass per ha in 4-5 months. When harvested green it is succulent and rich in celluloses and hemicelluloses, making it amenable for microbial digestion and fermentation (Ibeto *et al.*, 2011). Due to its short growing period, low water requirement, and large amount of alcohol produced, sweet sorghum have been strongly recommended as a main alcohol crop in Taiwan (Sin & Chien, 2009).

### 4.6 Sago starch as carbon source

Sago palm is important economic specie and is now grown commercially in Malaysia, Indonesia, Philippines, and New Guinea for the production of sago starch (Saifuddin &

Hussain, 2011). Sources of sago starch can be preparing from several genera of palms, Metroxylon, Borassus, Arenga and Cycads from the genus Cycas. They usually exist in the form of small whitish, pinkish and brownish. According to International Starch Institute, sago palm is mature and ready for harvest in 6-8 years. A mature sago palm can be 24-26 feet high and 17-27 inch thick.

In Malaysia, the use of sago starch has been increasing, and it is presently being used for the production of glucose. Sago starch represents an alternative cheap carbon source for fermentation processes that is attractive out of both economic and geographical considerations (Abd-Aziz, 2002).

The most important economic factor in fermentation is the cost of substrate, which made up about 60% of the overall cost of production (Ennis *et al.*, 1986). Hence, the availability of an inexpensive raw material such as sago starch is essential if fermentation is become to economically viable (Liew *et al.*, 2006). Generally, ethanol fermentation relies on sugar-rich substrates, mainly sugarcane and corn, because their carbohydrate is in fermentable form. However, sugarcane and corn is expensive material (Abd-Aziz *et al.*, 2001) if compare with sago starch.

On the other hand, carbon sources such as sugarcanes and corns are seasonal crops and not continuously available. While sago starch processing industries are abundant and readily available in Sarawak, East Malaysia (Flach, 1997). In Sarawak, it has been estimated approximately 7 tons of sago pith waste was produced daily from a single sago starch processing mill (Bujang *et al.*, 1996). Furthermore, as reported by Saifuddin & Hussain, 2011, using sago starch also has the possibility to have ethanol conversion efficiencies of up to 72%

(v/w). Taking an optimistic yield of 20 tons of clean starch per hectare, this comes down to an alcohol yield of 14, 400 litres per acre. Hence making sago starches is one of the most productive energy crops. Moreover, production of grain-based ethanol and vegetable-oil based bio-fuel is today facing difficulties due to competition with food supply (Demirbas, 2009); sago starch currently is not widely consumed as staple food. Therefore, the moral issue against food security can be reduced (Maherawati & Sholahuddin, 2010).

### 4.7 Batch- and repeated batch fermentation

The term of "batch" in fermentation is a type of closed system generally refer to both batch and fed-batch fermentation. In batch fermentation, all ingredients use in fermentation is fed inside the processing vessel at the beginning of the fermentation and no additional or withdrawal of material takes places during fermentation while new ingredients can be added during the batch run is called fed-batch fermentation. According to Cinar *et al.*, (2005), Batch processes have received increasing attention in the second half of the twentieth century. One of the reasons is that detailed process models are not available. Other advantages conferred to, batch processes are easier to set up and operated with limited knowledge, low maintains and low risk of contamination when compared to continuous processes, because if this advantages batch system is the favourite process in many fermentation industries. In our study we are proposing the use of repeated-batch fermentation method. The repeated-batch fermentation process combines the advantage of batch and fed-batch fermentation processes mainly making possible to conduct the process by long periods and improving the productivity compared to batch process (Treichel *et al.*, 2010). This statement supported by Yamakawa *et al.*, 2010 who reported that to reduce the cost of fermentation process and enhance ethanol

productivity through the use of high cell density, using repeated batch fermentation. Last but not least, from an industrial point of view by using the repeated batch mode the production period can be shortened, compared to standard fed-batch or batch processes resulting in a significant increase of the final product yield (Russ *et al.*, 2007).

## 5.0 MATERIALS AND METHODS

### 5.1 Yeast preparation and culturing

*Candida tropicalis* ATTCCa isolated from rotten pineapple was used in this study and it was kept at -84°C in yeast extract-glucose medium in 2 ml eppedendorf vials. One vial containing 1 ml of frozen yeast was thawed at room temperature and refreshed in 5 ml culture medium containing 20 g/L glucose, 5 g/L yeast extract. The broth culture was incubated for 9 h at 32°C. Sub-culturing was performed every 2 weeks.

### 5.2 Sago starch hydrolysis

The hydrolysis of sago starch has been reported elsewhere (Carvaval-Zarrabal *et al.*, 2008). The hydrolysis of sago starch was performed using enzymes from Novozyme with the conditions reported by the manufacturer. Briefly, 200 g of sago starch (dry basis) was suspended and dissolved in tap water. The final volume adjusted to 1 L. The pH of the suspension was adjusted to 6.5 and 1 µl of enzyme termamyl SC (Novozyme Co.) per gram of starch was added for liquefaction of starch at 90-95°C for 2 hours. The saccharification

process was performed by adding 1 µl Dextrozyme (Novozyme Co.) per gram of starch at pH 4.5, heated at 60-63 °C for 24h and agitated at 200 rpm.

### 5.3 Inoculum preparation

One tube containing refreshed active culture of yeast after 9 hours incubation was inoculated into 250 ml of Erlenmeyer flasks which contains 20 g/L glucose and 5 g/L yeast extract. The cells were cultivated on an orbital incubator shaker GYROMAX™ 706 at 100 rpm at 32°C for 17 hours. After 17 hours, the culture broth was centrifuged on high speed refrigerated centrifuge CR21G at 4000$g_c$ for 5 minutes to harvest the cells.

### 5.4 Batch and repeated batch fermentation

#### 5.4.1 Effect of the temperature on the growth of *Candida tropicalis*

The fermentation was carried out in 3 L Jar fermenter, fully computer controlled system. The parameters such temperature, pH, agitation, cell concentration, carbon dioxide production, were monitoring on-line in real time. Initially the fermentations were carried out in batch mode to study the effect of temperature on the growth of the isolated *Candida tropicalis*. For these experiments, commercial glucose and yeast extract were used as carbon and nitrogen sources at level of 30 g/L and 5 g/L respectively. The temperatures tested were 42°C and 45°C; the agitation was controlled at 200 rpm and without pH control. The inoculums size in this experiment was set at an optical density (OD) in the range of 0.045± 0.015 for the initial

fermentation. All the experiments at least were carried out by duplicate to report the mean of the results obtained the parameters monitored.

### 5.4.2 Repeated Batch Fermentation

The fermentation was performed at the same conditions as batch fermentation but the commercial glucose was replaced by hydrolyzed sago starch concentration as 30 g/L, 40g/L, 50g/L, 60 g/L, 90g/L and 100 g/L.

### 5.5 Analytical methods

During the batch fermentation, 5 ml of broth samples were collected every 3 hours for cell counts by the Hemacytometer method using a Hirschmann Laborgerate hemacytometer and reported as Colonies Forming Units (CFU). Briefly, 20 µl of samples and 20 µl of Methylene blue were mixed well in 1.5 ml of eppendorf during 5 minutes. From the eppendorf tube, 10 µl of mixed solutions were transferred into both sides surface of counting chambers and the cells were viewed under microscope. Blue cells were died and living cells were not stained. A standard curve of CFU/ml against OD was determined.

During RBF, the residual glucose concentration was determined by 3, 5-Dinitrosalicylic acid (DNS) method using Shimadzu UV-Vis Spectrophotometer model UV Mini-1240 to determine OD of glucose at the wavelength 575 nm. Ethanol concentration was determined by injected 25µl of samples into Shimadzu High-Performance Liquid Chromatography

(HPLC) system model LC-20AT equipped with four pumps and water/sulphuric acid (99: 1, v/v) as mobile phase with the flow rate set at 0.8 ml/min. The dry cell weight (DCW) was determined by a pre-established calibration curve of Optical density (OD) with the absorbance 575 nm against DCW. DCW was determined by which 2 samples of 50 ml from fermented broth were collected at the beginning and final of the fermentation and filtered using filter membrane pore size 0.45μm. The membrane was dried inside in an oven at 60°C for 72 hours and the weight was registered and plotted against the OD. The OD values were obtained from Turbidimeter Controller model LA-300LT (Tokyo Japan). It was determined that one OD unit wasequivalent to 6.4 g/l DCW.

### 5.6 Statistical Analysis

T- Test was used to compare means at the 5% significance level using statistic package Systat 7.0.

## 6.0 RESULTS AND DISCUSSIONS

### 6.1 Effects on the growth of *Candida tropicalis* at 42°C and 45°C

Figure 1 showed the effects of temperature of fermentation on biomass production at 42°C and 45°C. During the fermentation, initially temperature of 37°C had been applied for both 42°C and 45°C to promote the cell growth for the first 3h of fermentation. According to Slapack *et al.* (1987), defined yeast can be classified as thermo-tolerant if they can grow at least 40°C. Based on the figure 1 above, isolated *Candida tropicalis* strains are able to grow at both 42°C and 45°C but had poor fermentation rate. Hence, we can conclude *Candida tropicalis* strain is considered as thermo tolerant yeast.

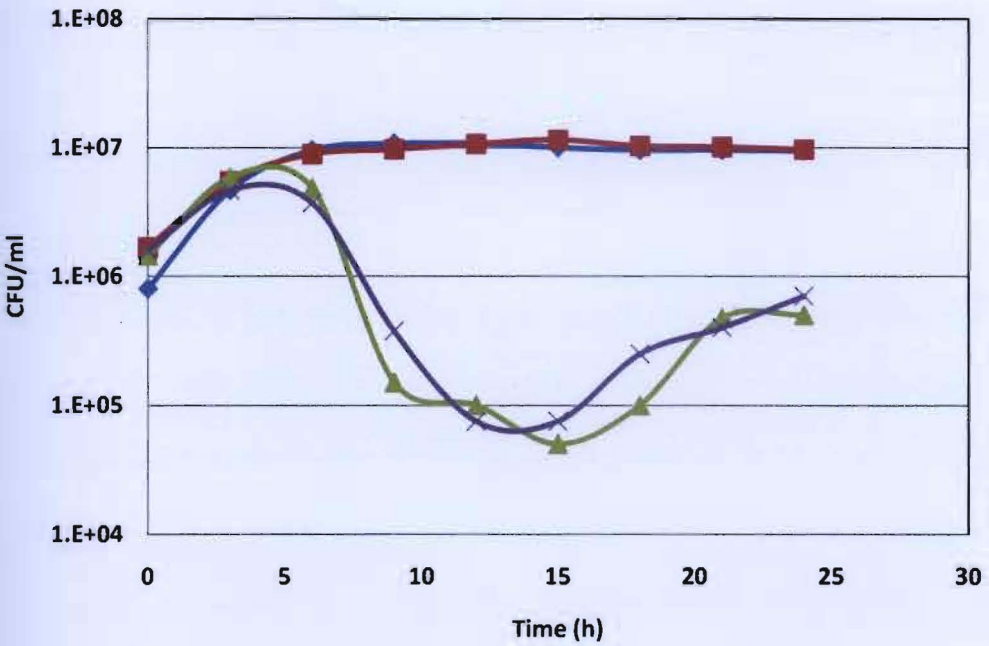


**Figure 1: Effects of high temperature on biomass production of *Candida tropicalis*. Symbols CFU/mL 42°C: Replicate 1(♦), (■) Replicate 2; CFU/ml 45°C: Replicate 1(▲), Replicate 2(×).**